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**ULTRASTRUCTURAL STUDY OF PROTECTIVE ENVELOPES
IN DIOECOCESTUS ASPER (CESTODA: DIOECOCESTIDAE)
MEGALOCERCUS**

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The megalocercus of *Dioecocestus asper* (Mehlis 1831) from the haemocoel of dragonfly larvae possesses two envelopes: outer (exocyst) and inner (endocyst) ones. The exocyst contains the large endocyst and larval strobila with scolex attached to the latter. Outer and inner surfaces of these envelopes are organized as the tegument and have some structural differences. The exocyst is covered with slender microvilli. Its outer tegument contains numerous mitochondria; the inner one is filled with lipid droplets released into the exocyst's cavity. The well-developed protonephridial (excretory) system consisting of flame cells, collecting ducts and canals is the unique feature of the exocyst, noted for the first time. Thick (more, than 50 µm) distal cytoplasm of the outer tegument of the endocyst is the place of accumulation of uniform globules looking like a hyaloid layer. This outer layer together with underlying fibrous layer (up to 20 µm), apparently, protect the scolex and larval strobila during the transfer through feather clump in the stomach of grebes, definitive hosts of *D. asper*. Muscle cells of both envelopes retain their synthetic activity even in the fully developed metacestode. Probably, they are the main structural element, which produces fibers of the extracellular matrix and maintains the integrity of protective envelopes of the megalocercus.

Key words: metacestode, ultrastructure, ascocercus, megalocercus, exocyst, endocyst, *Dioecocestus asper*.

**УЛЬТРАСТРУКТУРНОЕ ИЗУЧЕНИЕ ЗАЩИТНЫХ ОБОЛОЧЕК
МЕГАЛОЦЕРКА DIOECOCESTUS ASPER (CESTODA: DIOECOCESTIDAE)**

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Мегалоцерк *Dioecocestus asper* из гемоцеля личинок стрекоз имеет 2 оболочки: внешнюю (экзобиостру) и внутреннюю (эндобиостру). Экзобиостра содержит крупную эндобиостру с прикрепленной к ней зародышевой стробилой со сколексом. Наружная и внутренняя поверхности обе-

их оболочек организованы как тегумент и имеют некоторые различия в структуре. Экзоциста покрыта тонкими микроворсинками; ее наружный тегумент содержит множество митохондрий, внутренний богат липидными каплями, которые высвобождаются в полость экзоцисты. Развитая протонефридиальная (экскреторная) система, состоящая из пламенных клеток, собирающих протоков и каналов является уникальной особенностью экзоцисты, которая отмечена впервые. Толстая (более 50 μm) дистальная цитоплазма наружного тегумента эндоцисты — это скопление однородных глобул, которое выглядит как стекловидный слой. Этот наружный слой вместе с подлежащим фиброзным слоем (до 20 μm толщиной), вероятно, защищают скоплекс и личиночную стробилу во время прохождения через перьевую пробку в желудке поганок, окончательных хозяев *D. asper*. Мышечные клетки обеих оболочек сохраняют синтетическую активность даже у полностью сформированной метацестоды. Вероятно, они являются основным структурным элементом, который производит волокна межклеточного матрикса и поддерживает целостность защитных оболочек мегалоцерка.

Ключевые слова: метацестода, ультраструктура, аскоцерк, мегалоцерк, экзоциста, эндоциста, *Dioecocestus asper*.

Metacestodes of *Dioecocestus asper* (Mehlis 1831) were found for the first time in the stomach of the red-necked grebe, *Podiceps griseigena* Boddaert 1783 in 1948 (Jögis, 1978). Their intermediate hosts, however, were determined much later (Gulyaev et al., 2010). Studies of metacestodes of *D. asper* from spontaneously infected hosts (Regel, Pospekhova, 2012; Regel et al., 2013) showed that the first finding (Jögis, 1978) represented the endocyst with enclosed prospective parts (scolex and strobila), whereas the outer envelope (exocyst) was apparently lost in the stomach of the red-necked grebe.

By present, postembryonic development of *D. asper* has been traced from the stage of the primary cavity to completely developed metacestode, which was named ‘megalocercus’ (Regel et al., 2013).

The formation of *D. asper*’s exocyst occurs during the first invagination, when the front pole of the metacestode with developing anlage of cystoscolex is merged inside. The invagination channel is closed, whereas outer and inner layers undergo differentiation. In the mature metacestode, they form an integrated envelope with morphologically different outer and inner surfaces. The endocyst is formed later after the second invagination, when the base of larval strobila is retracted almost as far as the back pole of the endocyst. As a result, another integrated envelope with morphologically different inner and outer layers is formed (Regel et al., 2013).

The present article represents results of the study of the protective envelopes of completely developed metacestode *D. asper* in optical and electron microscopes.

MATERIAL AND METHODS

The original material was obtained by dissection of dragonfly larvae (Anisoptera) of the genus *Aeshna* from lakes of the Upper Kolyma basin. Metacestodes were fixed in 2 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at about 4 °C. After fixation the material was additionally fixed in a 2 % OsO₄ in 0.2 M phosphate buffer (pH 7.2) for 12 hours, dehydrated and embedded in a EPON-araldite mixture. During dehydration, specimens were stained with saturated solution of uranyl acetate in 70 % ethanol by night. Ultra-thin sections (90 nm), obtained with the use of LKB Bromma 2088 and LKB Nova ultratomes (Sweden), were viewed in transmission electron microscopes JEM-1011

(JEOL, Japan) operating at 80 kV and Libra-120 (Carl Zeiss, Germany) operating at 120 kV. Semi-thin sections (1—2 μm) were stained with methylene blue using the method of Morgenstern (1969) and analyzed and photographed in optical microscopes Biomed-2 (Biomed, Russia) with a Canon Power Shot A95 digital camera (Canon Inc., Japan) and an Olympus CX41 (Olympus Corporation, Japan) with an Olympus E-420 digital camera.

RESULTS

In an optical microscope, the outer envelope (exocyst) of living *D. asper* looks like a thin-walled translucent sack. At early developmental stages, the exocyst is considerably larger comparing to anlage of cystoscolex (fig. 1, *a*, see inset); at the mature metacestode it covers endocyst with long actively moving strobila and scolex (fig. 1, *b*). After removing of the outer envelope, it can be seen that the surface of endocyst is formed by the layer of hyaloid transparent material.

Average thickness of exocyst at semi-thin sections is 20 μm . Outer and inner surfaces of exocyst are covered with microvilli; the outer surface has deep folds, whereas the inner one is relatively flat (fig. 1, *c*). The outer layer of the exocyst under microvilli looks dense; thin muscle fibers lie beneath this layer; deeper, processes filled with dense polymorphous bodies are found, and, finally, cell bodies with large nuclei and dense nucleoli. The inner layer of the exocyst is organized in a similar way; muscle fibers, however, are larger in cross-section, cell bodies are not so numerous, and cell processes have round dense inclusions. Bodies of muscle cells are usually located in the depth of the exocyst. Excretory canals are nearer to the inner surface (fig. 1, *c*).

The surface of the endocyst has a helical relief, in semi-thin sections resembling teeth directed forward (fig. 1, *d*). The outer transparent layer is intensively stained, showing its different thickness. It is minimal at the inner surface of teeth, reaching 50 μm between the teeth. On the back pole of the endocyst, its surface layer gradually becomes thinner, and in the area of the excretory pore it smoothly turns into excretory atrium lining (fig. 1, *e*).

Two fibrous layers with mutually perpendicular orientation of fibers, 15 μm thick on the average, lay under the outer surface of the endocyst. The inner fibrous layer is contiguous with muscle fibers of small diameter (fig. 1, *f*). Beneath this layer, there are cellular elements, among which muscle cells can be identified. They are situated closely to multiple calcareous bodies, scattered among processes without visible filling. The latter form the basis of medullary part of the endocyst. Large excretory canals are located closer to the inner surface of the endocyst, neighboring sparse cellular elements. Subsurface musculature is well developed, being formed of circular and longitudinal fibers about 2 μm in diameter.

Electron microscopic study of the exocyst shows that its outer and inner surfaces are the tegument, covered with a layer of 3—6 μm long microvilli (fig. 2, see inset). The tegument of the outer surface (fig. 2, *a, b*) (referred below as the ‘outer tegument’) possesses dense distal cytoplasm filled with clear vesicles. The presence of numerous mitochondria that also fill processes connecting cytons with distal cytoplasm is its peculiar feature. Basal matrix is thin, but, direct-

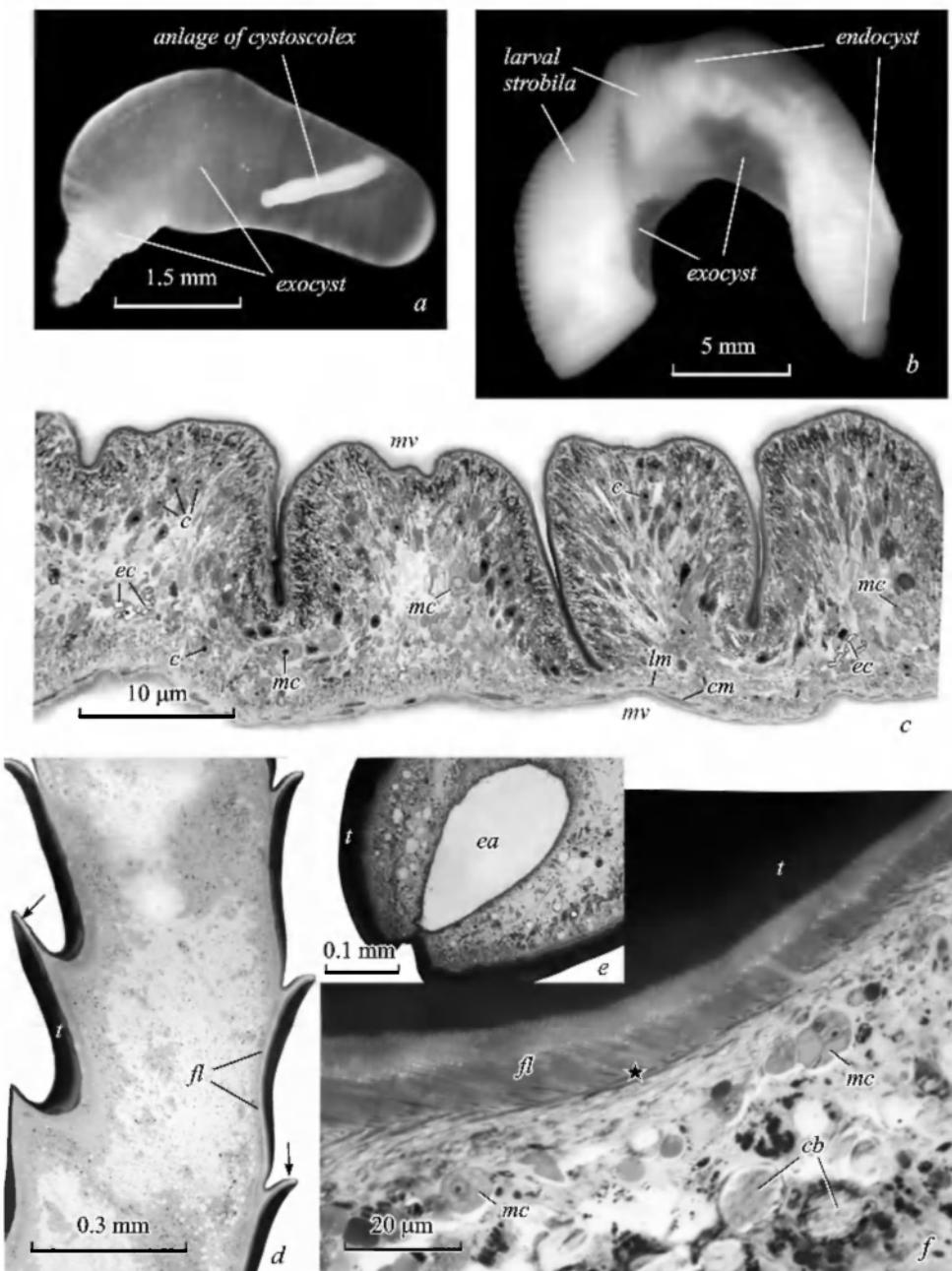


Fig. 1. Light microscopy of the protective envelopes in *D. asper* metacestode.

a — living metacestode after first invagination; *b* — fully developed living metacestode; *c* — semi-thin section of exocyst showing tegumental cytons (*c*), circular muscle (*cm*), excretory canals (*ec*), longitudinal muscle (*lm*), muscle cells (*mc*) and microvilli (*mv*); *d* — semi-thin section of endocyst's crust showing the «teeth» of spiral relief (*t*), hyaloid layer (*t*) and fibrous layer (*fl*); *e* — back pole of endocyst with excretory atrium (*ea*) and hyaloid layer (*t*) (semi-thin section); *f* — outer part of endocyst showing hyaloid (*t*), fibrous (*fl*), muscle (asterisks) layers and also calcareous bodies (*cb*), and muscle cells (*mc*) (semi-thin section).

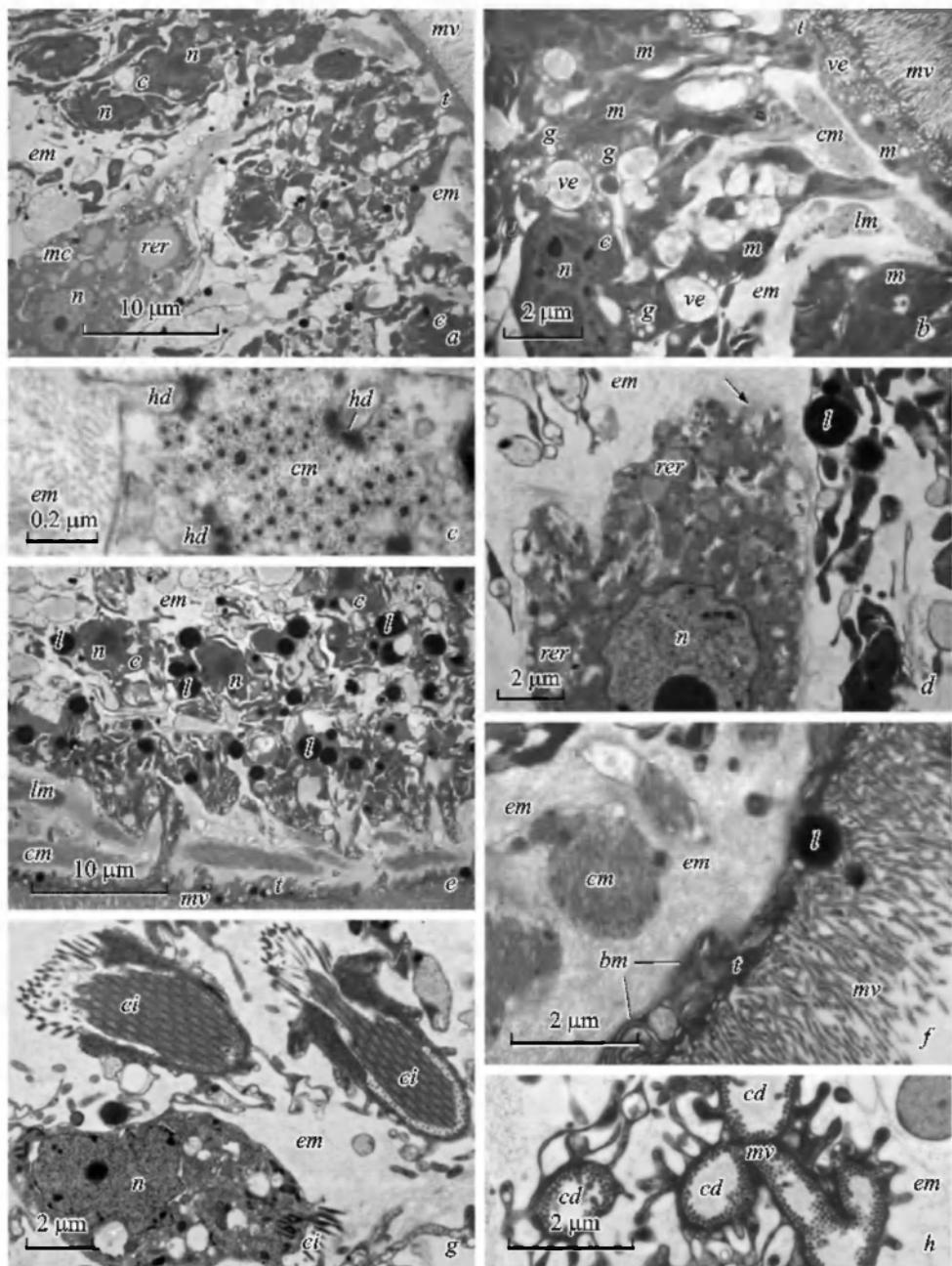


Fig. 2. Ultrastructure of the exocyst in *D. asper* metacestode.

a — outer tegument, b — detail of outer tegument, c — circular muscle of outer tegument, d — muscle cell (arrow shows the bundle of fibrous material), e — inner tegument, f — detail of inner tegument, g — flame cells, h — collecting ducts. Abbreviations: *bm* — basal matrix, *c* — cyton, *cd* — collecting duct, *ci* — cilium, *cm* — circular muscle, *em* — extracellular matrix, *g* — Goldy zone, *hd* — hemidesmosome, *l* — lipid droplet, *lm* — longitudinal muscle, *m* — mitochondrion, *mc* — muscle cell, *mv* — microvilli, *n* — nucleus, *rer* — dilated cisternae of rough endoplasmic reticulum, *t* — distal cytoplasm of tegument, *ve* — vesicle.

ly under it, there is a fibrous extracellular matrix of the exocyst, into which both subtegumental musculature and cellular elements of the exocyst are submerged. Tegumental cytons have signs of high synthetic activity: long channels of the rough endoplasmic reticulum (RER) and plenty of Golgi zones surrounded with vesicles. Circular muscle fibers of the outer surface are 1.2—1.5 μm in diameter. Longitudinal fibers lie slightly deeper; they have the same thickness and are arranged apart from each other. Processes going from muscular cells to contractile parts contain vesicles and mitochondria. Musculature is attached to the surrounding extracellular matrix by hemidesmosomes (fig. 2, c).

Large muscle cells (up to 15 μm in diameter) with expanded RER cisternae are usually located a bit deeper than tegumental cytons (fig. 2, a). Cytoplasmic membrane of cells frequently contacts with bundles of fibrous material, which is abundant in hollows (fig. 2, d).

Cytons of the inner surface of the exocyst produce vesicles with granular material and contain a lot of lipids that pass to distal cytoplasm together with vesicles and are released into the inner cavity of the exocyst (fig. 2, e, f). Mitochondria are noted in areas with less content of lipid drops. Subtegumental musculature of the inner surface of the exocyst consists of circular fibers, 2—2.5 μm in diameter, and longitudinal fibers of similar diameter, which do not form a continuous layer.

Elements of the excretory system (flame cells, collecting tubules, and excretory canals) are located closer to cytons of the inner surface (fig. 2, g, h).

Electron microscopic study of endocyst of *D. asper* (fig. 3, see inset) showed the outer transparent layer to be the distal cytoplasm of the tegument filled with light tightly packed globules, 120—150 nm in diameter (fig. 3, a, b). Their shape changes from round to angular (fig. 3, c).

The surface of the tegument is covered with a reticulate glycocalyx. They both are 50 μm thick; there are two massive fibrous layers with mutually perpendicular oriented fibrils under them (fig. 3, a, d). Small in section circular and longitudinal muscle fibers, 0.5—1 μm , are situated close to the inner fibrous layer (fig. 3, d). They are regularly accompanied by muscle cells with expanded RER cisternae (fig. 3, e). Medullar zone of the endocyst is filled with multiple membrane profiles without visible content, among which there are calcareous bodies.

Elements of excretory system (flame cells, collecting ducts and large canals) are located nearer to the inner surface of the endocyst, though some collecting ducts are noted in the entire thickness of the endocyst beginning from the cellular layer of the outer tegument (fig. 3, f).

Scattered cytons of the inner tegument have vacuolated cytoplasm and condensed chromatin (fig. 3, g). Subtegumental musculature of the inner tegument consists of circular and longitudinal layers. The diameter of circular and longitudinal fibers constitutes 1—2.2 μm and about 1.5 μm , respectively. Muscle cells are located somewhat deeper (fig. 3, g). There are some poorly differentiated muscle cells of rounded shape with small tubules of RER among them (figs. 3, h). The inner surface of the endocyst is slightly sinuous, microvilli are short and sparse. Distal cytoplasm is poorly developed; in some cases, it contains only vesicles with flaky material excreted into the cavity of the endocyst. Sparse microtrichia appear in basal part of the endocyst in the attachment area of the larval strobila retractor (fig. 3, i).

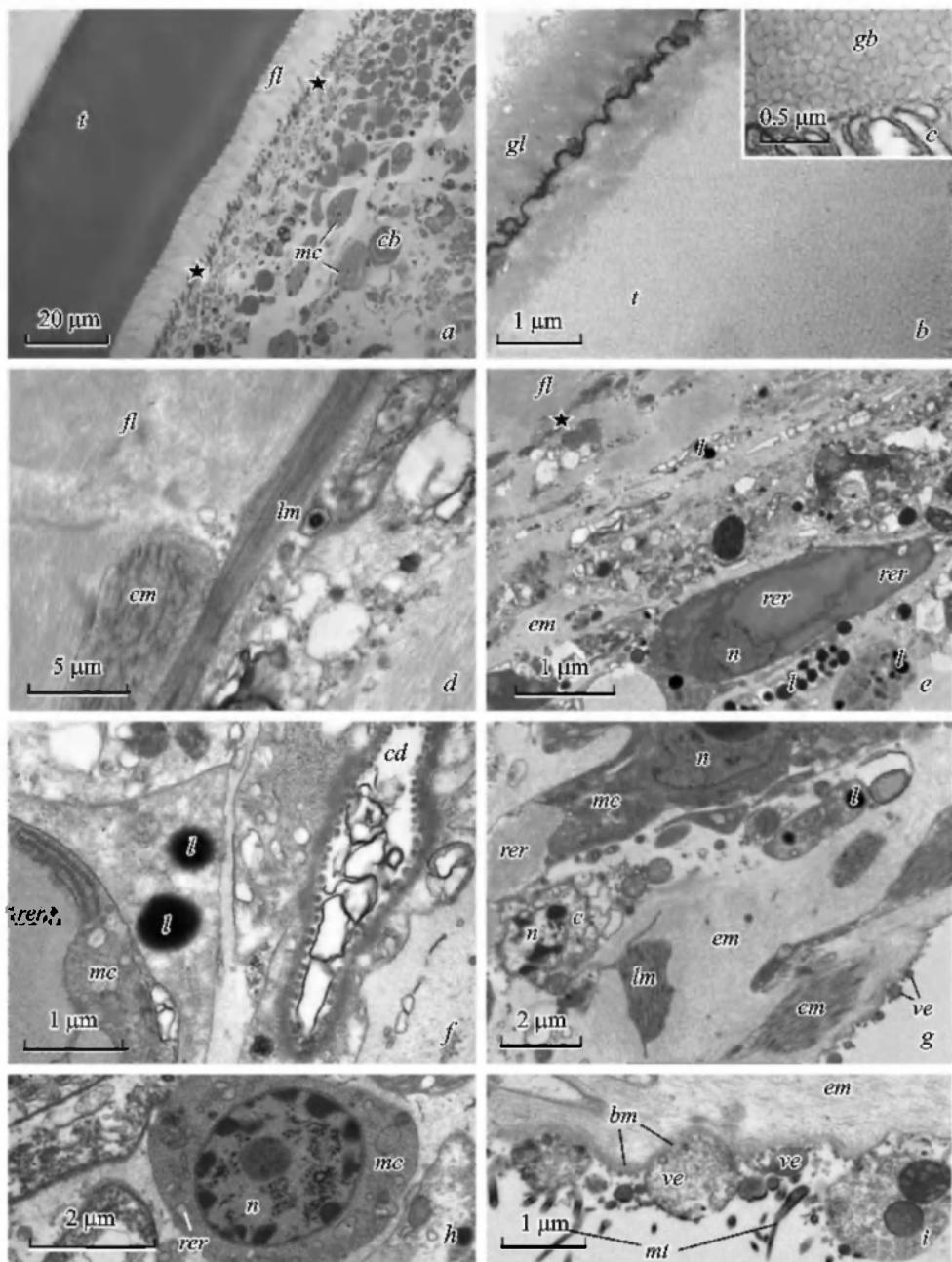


Fig. 3. Ultrastructure of the endocyst in *D. asper* metacercyste.

a — outer tegument of endocyst, *b* — outer part of its distal cytoplasm, *c* — inner part of distal cytoplasm, *d* — fibrous and muscle layers, *e* — muscle cell near these layers, *f* — collecting duct, *g* — inner tegument of endocyst, *h* — poorly differentiated muscle cell, *i* — inner tegument in basal part of endocyst. Abbreviations: *bm* — basal matrix, *c* — cyton, *cb* — calcareous body, *cd* — collecting duct, *cm* — circular muscle, *em* — extracellular matrix, *fl* — fibrous layer, *gb* — globules, *gl* — glycocalyx, *l* — lipid droplet, *lm* — longitudinal muscle, *mc* — muscle cell, *mt* — microtriches, *mv* — microvilli, *n* — nucleus, *rer* — rough endoplasmic reticulum, *t* — distal cytoplasm of tegument, *ve* — vesicle. Black asterisk — muscular layer.

On the back pole of the endocyst, distal cytoplasm of the outer tegument becomes thinner, being 30 μm thick in the area of excretory pore and 10 μm directly after entering into excretory atrium (fig. 4, *a*, see inset). In the distal cytoplasm of the excretory pore, there are small cavities with loose material and dense drops especially numerous near the outer membrane. In this region, the glycocalyx has inclusions of dense material and vesicles (fig. 4, *a*, insert).

The tegument of the excretory atrium has reticulate glycocalyx, 0.5—1 μm thick (fig. 4, *b*). Vesicles and fragments of loose material, 0.3—0.8 μm in diameter, can be seen near its surface in the lumen of the atrium (fig. 4, *b*, *c*). Distal cytoplasm, 3—4.5 μm thick, has deposits of dense material under the outer membrane and contains plenty of globules and twisted small bodies, 100—120 nm thick and up to 1 μm long. Close to the distal cytoplasm there are large processes, up to 4 μm in diameter, containing both twisted small bodies and separate globules of the same diameter (fig. 4, *b*, *c*). Circular and longitudinal muscular fibers, 1—2 μm in diameter, are located under the basal matrix among extracellular matrix. Cellular elements do not constitute a solid layer and have no evident signs of high functional activity. Membrane profiles and fragments of degenerated cells regularly occur in the area of cellular bodies (fig. 4, *b*).

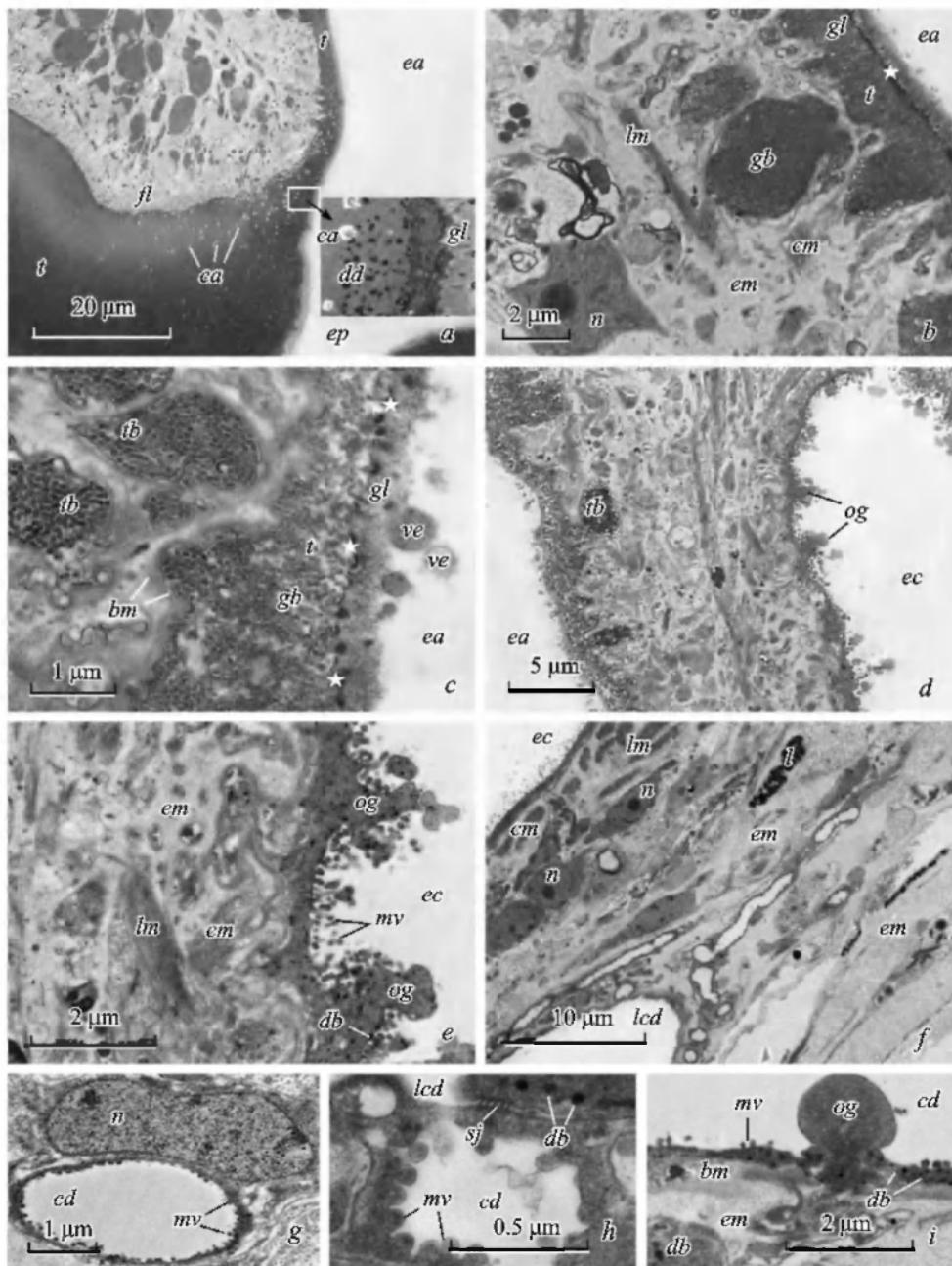
The tegument of the main excretory canals is covered with round microvilli and possesses numerous outgrowths near the place of the confluence with the excretory atrium (fig. 4, *d*, *e*). The distal cytoplasm is about 0.5 μm thick; the length and the diameter of outgrowths constitute 2 to 5 μm and up to 1.5 μm , respectively. They are formed by the material of the distal cytoplasm and contain in the same discoid bodies as the latter (fig. 4, *e*). The bodies come from cytons underlying the basal matrix and muscle fibers.

Distal cytoplasm of main excretory canals remote from the atrium has no outgrowths, its microvilli are more stretched (fig. 4, *f*). Cytons of excretory canals underlie basal matrix and muscle fibers, which form two layers — circular and longitudinal ones. Small collecting ducts are formed by individual cells (fig. 4, *g*) and are attached to larger ones by septate junctions (fig. 4, *h*). The walls of collecting ducts are covered with round microvilli with a dense core and have rare rounded outgrowths (fig. 4, *i*).

DISCUSSION

Comparison of morphology of protective envelopes in alive and embedded into epoxy medium *D. asper* metacestodes demonstrated differences in linear sizes both in the whole envelopes and in their components. It can be explained by muscle contraction during fixation and also by the total tightening of tissues during preparation of material for electron microscopic study. Thus, in alive metacestodes, the exocyst is 9—10 μm thick (Regel et al., 2013), whereas in semi-thin sections, the folded exocyst is 20 μm and more thick. Endocyst has less fluctuating thickness, apparently, due to high density of the outer transparent layer (i. e. distal cytoplasm of the tegument).

At present, only a single ultrastructural study of the protective envelopes of metacestode of the similar structure is known (Rees, 1973); later, V. D. Gulyaev suggested to name it the ‘ascocercus’ (Gulyaev, 1998). The ascocercus is cha-

Fig. 4. Ultrastructure of the back pole of the endocyst in *D. asper* metacestode.

a — tegument of excretory pore and excretory atrium (insert — the enlarged fragment), b — tegument of excretory atrium, c — distal cytoplasm of tegument of excretory atrium, d — excretory atrium and main excretory canal, e — tegument of main excretory canal near the excretory atrium, f — excretory canal and collecting ducts remote from atrium, g — duct cell, h — attachment of small collecting duct to larger one, i — round outgrowth of tegument of collecting duct. Abbreviations: bm — basal matrix, c — cyton, ca — cavity with loose material, cd — collecting duct, cm — circular muscle, db — discoind body, dd — dense droplet, ea — excretory atrium, ec — excretory canal, em — extracellular matrix, ep — excretory pore, fl — fibrous layer, gb — globules, gl — glycocalyx, l — lipid droplet, lcd — large collecting duct, lm — longitudinal muscle, mv — microvilli, n — nucleus, og — outgrowth, t — distal cytoplasm of tegument, tb — twisted body, ve — vesicle, white asterisks — the dense material under the limiting membrane of tegument.

racterized by two protective envelopes: the outer cellular envelope (the exocyst) and inner envelope (the endocyst), into which the scolex is withdrawn. Diplocysts of some Aploparaksidae also possess cellular exocyst, but it is constantly connected with the inner envelope (Nikishin, 2010). Exocyst of the ascocercus is significantly bigger than the cystoscolex anlage that is separated from the exocyst and freely moves in its cavity (Rees, 1973; Gulyaev, 1998). In megalocercus, this trait is visible only at early stages of postembryonic development. The exocyst of fully developed metacestode *D. asper* is filled with the large endocyst and larval strobila with scolex. However, the general structural scheme of the ascocercus and the megalocercus is similar. This, together with the usage of the same, evolutionary ancient group of intermediate hosts (Odonata), suggests that they are assigned to the same morpho-ecological group of cysticercoids (Regel, Pospekhova, 2012; Regel et al., 2013). Probably, *Schistotaenia tenuicirrus* Chandler, 1948 metacestode applies to the same group. It is comparable in size to *D. asper* megalocercus, it has a voluminous cellular exocyst and a separate endocyst with larval strobila and scolex, and, finally, it develops in big dragonflies (Boertje, 1975). The author has named this metacestode the «strobilocercoid» by analogy with the strobilocercus of some Taeniidae, but we believe that the presence of larval strobila is less important feature than the uniform structure and development.

Ultrastructural study of the outer envelope of *Tatria octacantha* Rees, 1973 cysticercoid showed peculiar arrangement of subtegumental musculature in the exocyst: only circular muscles are found in the outer side and only longitudinal ones, in the inner side (Rees, 1973). In the case of *D. asper*, circular and longitudinal muscle fibers occur both in the outer and inner subteguments. Such muscular organization corresponds to the formation process of the double-layer exocyst through invagination of the same tegument. Massive musculature of *D. asper*'s exocyst can be explained by large size of the metacestode, while morphological differences of the outer and inner teguments, by specialization of once uniform cover to different functions. Judging by the number of mitochondria, the outer tegument is specialized to the active transport of substances. Additionally, its surface also participates in the secretion of material. The inner tegument is also involved in secretion; its more prominent function is, however, the transport of numerous lipid drops to distal cytoplasm and their delivery into the exocyst's cavity. Possibly, lipids serve as reserve material used by the large larval strobila of the metacestode.

Regarding excretory system of the exocyst of *D. asper*, it has no analogues among metacestodes with the cellular exocyst. Apparently, the presence of a well-developed excretory system with flame cells, collecting ducts, and canals can be explained by the large size of the metacestode and considerable thickness of the outer envelope.

General structural scheme of the endocyst of *D. asper* is similar to that in representatives of Hymenolepididae and Aploparaksidae (Krasnoshchekov, Nikishin, 1979). The endocyst consists of the tegument, covered with reticulate glycocalyx, lying beneath fibrous layers and the cellular layer. The arrangement of muscular fibers in *D. asper* endocyst (the latter is situated under fibrous layers) and the structure of the distal cytoplasm filled with separate globules remind endocyst's structure in representatives of Dilepididae (Krasnoshchekov et al., 1983). At the same time, the endocyst of the fully developed *D. asper* meta-

cestode has a unique structure, differing from that in other representatives of cystomorphous metacestodes. First of all, the endocyst tegument, with its significant thickness and massive homogenous accumulation of transparent globules, is unusual. Apparently, these globules (or their precursors) are produced by cytons of the tegument during or after the second invagination. Anyway, during the second invagination, the endocyst preserves plasticity, which allows invaginating the front part of the endocyst with the attached larval strobila. In mature *D. asper* metacestode, the endocyst is a dense structure resembling insect pupa (Regel et al., 2013). Probably, the process of its hardening is associated with maturing and tight arrangement of the secrete of tegumental cytons (twisted bodies and globules) in distal cytoplasm of the outer tegument of the endocyst. Later, tegumental cytons undergo degradation and a lot of degenerated cells are determined in the cellular layer of the mature metacestode.

Seemingly, the tegument of the excretory atrium is formed somewhat later than the outer tegument of the endocyst, and it has the similar structure. This assumption is confirmed by the content of large processes, approaching the tegument of the atrium, with twisted bodies and globules morphologically similar to those found in the outer tegument of the endocyst. Cells producing these bodies and globules were not found; however, remains of degenerated cells among unidentified cellular elements near atrium's tegument were observed.

Muscle cells in both exocyst and endocyst of mature metacestode show high synthetic activity. Apparently, this is rather associated with the production of fibers that form the extracellular matrix of both envelopes, than with supporting of contractile elements. Muscle cells are the largest cellular elements of the exocyst. Their surface is covered with bundles of thin fibers possibly included into the extracellular matrix of the exocyst.

The outer tegument of the endocyst is underlaid with rather massive fibrous layers that increase mechanical strength of the outer tegument. Muscle cells with broadened cisternae of RER, that can occupy the most part of the cell, are situated near fibrous layers. Moreover, young muscle cells of round shape with poorly developed RER can be found in the depth of the endocyst. Muscle cells were found near excretory canals; their structure is typical of the sunken epithelium, including basal matrix and subtegumental musculature.

Probably, fibrous component of both envelopes is very important for supporting of their integrity.

It is supposed, that functional significance of the outer envelope (the exocyst) includes protection of developing endocyst and prospective part of metacestode from influence of immune mechanisms of the intermediate host (Krasnoshchekov, 1980). Structural peculiarities of the exocyst of *D. asper* suggest that the exocyst is involved in active transport of nutrients into the inner cavity and in protection of the prospective parts — scolex and strobila. Functional significance of the outer transparent layer and underlaid fibrous layer of endocyst includes mechanical protection of the metacestode during passage through feather clump in the stomach of grebes (Simmons, 1956), definitive hosts of *D. asper*. On the other hand, it is hardly probable that the dense thick wall of the endocyst can provide the transport of substances to the developing strobila. Apparently, this very fact explains the presence of the prospective part of *D. asper* in exocyst cavity (not in the endocyst!), and, besides, the presence of the long larval strobila, that takes the function of nutrients fixation.

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